Purification and renaturation of recombinant proteins produced in *Escherichia coli* as inclusion bodies

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Key words: *Purification • recombinant proteins • inclusion bodies • E. coli • gel filtration • ion exchange • HIC*

Introduction

Expression of eukaryotic genes in the cytoplasm of *Escherichia coli* often leads to the production and accumulation of recombinant protein (*r*-protein) to levels of total cell protein that are higher than when secreted (1), in some instances by as much as 25%. The *r*-proteins so produced are frequently in the form of insoluble aggregates (2, 3), so-called inclusion bodies.

The inclusion bodies contain reduced and denatured proteins lacking biological activity (4, 5). Their sizes and densities are distinct from most of the bacterial proteins (6, 7), which facilitates their separation from intracellular *E. coli* proteins and cell debris by slow-speed centrifugation (4). A general scheme for extraction and purification of inclusion bodies from *E. coli* cells is shown in Figure 1. The purified inclusion bodies are usually dissolved in 6 M guanidine hydrochloride or 8 M urea at near neutral pH prior to renaturation and purification of the *r*-protein.

The most commonly used procedure for refolding of such denatured *r*-proteins is slow dialysis, or dilution into a buffer of near neutral pH (8). This results in significant dilution of the *r*-protein, typically to a few milligram of *r*-protein per milliliter of solution, with the formation of precipitated proteins often being unavoidable. Alternative purification and/or refolding procedures using gel filtration (GF), ion exchange (IEX), or hydrophobic interaction chromatography (HIC) have been developed to circumvent such problems. These procedures are outlined briefly in this Application note.

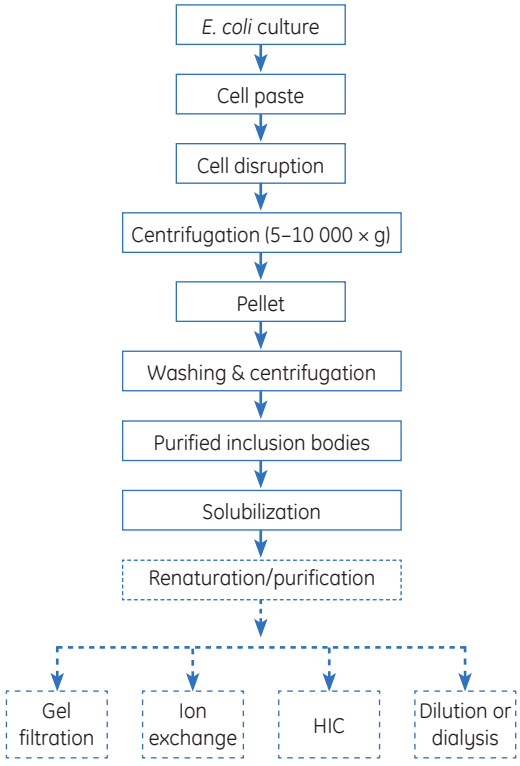

Fig 1. General scheme for extraction, solubilization, and renaturation (refolding) of eukaryotic proteins (recombinant proteins) produced as insoluble inclusion bodies in the cytoplasm of *Escherichia coli* cells. In some instances, the procedure used for refolding can result in significant purification of the solubilized and denatured recombinant protein. The dotted squares indicate alternative methods for the refolding process.

Procedures using gel filtration

Gel filtration of solubilized inclusion bodies in 6 M guanidine-HCl or 7 to 8 M urea has been used by many researchers (9–12) to purify the desired *r*-protein in its denatured state. Gel filtration media such as Sephadex™ G-100, Sepharose™ CL-6B, Sephacryl™ S-200, S-300 and S-400, and prepacked Superose™ 12 have successfully been used to remove extraneous protein impurities and polymeric forms of the *r*-protein. The purified, but still denatured, *r*-protein is then refolded by dialysis or dilution. A typical example is described below.

Methodology

Recombinant human interleukin-2 (IL-2) produced in *E. coli* was purified by Weir and Sparks (10) as follows:

One part of the inclusion bodies was suspended in one part 8 M guanidine-HCl, 10 mM DTT (dithiothreitol), pH 8.5, and incubated for 1 h at 37°C to ensure complete reduction and solubilization. An aliquot (approx. 200 µl) was applied to a prepacked Superose 12 HR 10/30* column (bed volume 24 ml) connected to FPLC™ System. The column was equilibrated and eluted with 6 M guanidine-HCl, 50 mM Tris-HCl, 10 mM DTT, pH 8.5, at a flow rate of 0.5 ml/min (linear flow rate 38 cm/h). Fractions of 1 ml were collected.

Preparative gel filtration was performed on a column packed with Sepharose CL-6B (5 × 90 cm, bed volume 1.8 l) using 20 ml of the reduced and solubilized inclusion bodies. The column was equilibrated and eluted with the buffer used above at a flow rate of 70 ml/h (linear flow rate 3.6 cm/h). Fractions of 9.5 ml were collected. The elution profiles obtained are shown in Figure 2. Fractions containing the IL-2 were pooled and renatured by dilution.

A similar procedure was used by Gill *et al.* (9) to purify recombinant chicken and bovine growth hormones, by Marciani *et al.* (11) to purify recombinant envelope protein of HIV, and by Fountoulakis *et al.* (12) to purify a soluble human interferon γ-receptor.

* HR 10/30 columns have been replaced by Tricorn high-performance 10/300 GL columns, see ordering information

Recommended media

For high-resolution gel filtration at analytical scale, we recommend Superdex™ Peptide, Superdex 75, and Superdex 200 (all 13-µm beads and prepacked in Tricorn™ high-performance columns). Suitable media for preparative gel filtration are Superdex 30 prep grade, Superdex 75 prep grade, Superdex 200 prep grade, Sephacryl S-100 HR, Sephacryl S-200 HR, and Sephacryl S-300 HR. These gel filtration media are rigid and can be used at relatively high sample loads and flow rates to give reproducible results in a short time.

Variation of technique

A very promising variant of the techniques outlined above was published by Werner *et al.* (13) in which refolding of the

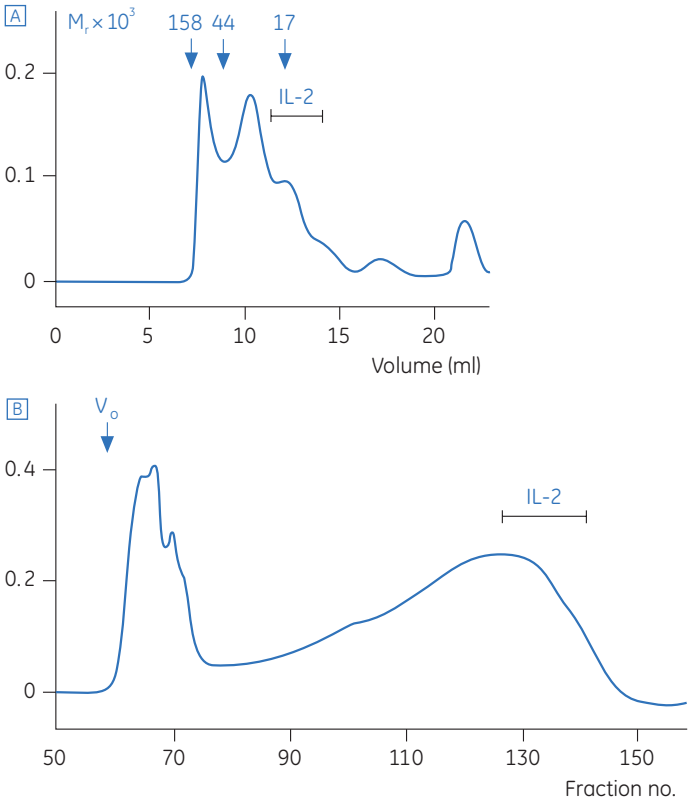

Fig 2. Gel filtration of pellet extracts. **A)** 200 µl (5.4 mg/ml) pellet suspension applied to Superose 12 HR 10/30* column. Molecular weight (M_r) markers were denatured gamma globulin (158 000), ovalbumin (44 000), and myoglobulin (17 000). **B)** 20 ml pellet extract (9.25 mg/ml) applied to Sepharose CL-6B column. (Reproduced with kind permission of author and publisher [10]).

r-protein was obtained during gel filtration of the solubilized and denatured inclusion bodies. The *r*-proteins were dissolved in 50 mM Tris-HCl buffer, 50 mM DTT, 200–500 mM NaCl, 6–8 M guanidine-HCl, pH 8.5, and applied to a column of Superdex 75 (HR 10/30) or Sephacryl S-100 HR (2.6 × 100 cm). For experimental details and general applicability of this procedure, see reference 12. The authors claim that "...the relatively high success rate using gel filtration may be that refolding and association on the column occurs under essentially irreversible conditions...".

Procedure using IEX or HIC

An interesting approach to simultaneous solubilization and renaturation of *r*-proteins from inclusion bodies in whole bacterial lysates was reported by Hoess *et al.* (14) using ion exchange or HIC media. Their procedure avoids the use of denaturing agents for solubilizing the inclusion bodies and leads to the refolding of the *r*-protein with biological activity comparable to that of its natural counterpart. The validity of their procedure was demonstrated using three *r*-proteins with molecular weight in the range of 15 000 to 45 000. They also conclude that adsorption of the *r*-protein to the ion exchange or HIC medium is not essential for solubilizing the *r*-protein from the inclusion bodies.

Methodology

In a typical experiment (14), the *E. coli* cell paste was suspended in 1/40 of the cell culture volume of buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM phenyl methyl sulfonyl fluoride [PMSF], 10% glycerol) containing 5 mg/ml of lysozyme. The suspension was allowed to stand for 15 min at 0°C and sonicated three times for 20 s. After standing for 15 min at 0°C, a 0.5-ml aliquot of the sonicated suspension was mixed with 0.5 ml of settled medium (Q Sepharose Fast Flow or SP Sepharose Fast Flow) that was equilibrated with buffer A. The mixture was shaken for 2 h at 4°C and centrifuged for 5 min at 14 000 rpm in an Eppendorf™ microcentrifuge. The supernatant was discarded and the pellet (containing insoluble material and the Q or SP Sepharose Fast Flow) was eluted with buffer A, containing 250 to 750 mM NaCl, by shaking for 15 min. The suspension was centrifuged and the supernatant, containing the solubilized and renatured *r*-protein, was characterized by immunological, electrophoretic, and biological assays.

Summary

Table 1 below summarizes the methods and media used in the purification and/or renaturation of *r*-proteins produced as inclusion bodies in *E. coli*.

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Table 1. Methods and media used in the purification and/or renaturation of *r*-proteins produced as inclusion bodies in *E. coli*

Purpose	*r*-protein	Method	Medium/column	Reference
Purification of *r*-proteins in the denatured state	Bovine growth hormone	GF in 6 M guanidine-HCl, pH 8.5	Sephacryl S-200 HR	9
	Interleukin-2	GF in 6 M guanidine-HCl, 10 mM DTT, pH 8.5	Superose 12 HR 10/30* Sepharose CL-6B	10
	HIV envelope proteins	GF in 6 M guanidine-HCl, 0.1% DTT, pH 9	Sephacryl S-300 HR	11
	Interferon γ-receptor	GF in 7 M urea, pH 7.5	Sephadex G-100	12
Refolding of *r*-proteins	Human ETS-1	GF in 20 mM HEPES, pH 6.5		
	Ribonuclease A	GF in 20 mM sodium phosphate, pH 7	Superdex 75 HR 10/30* Sephacryl S-100 HR	13
Simultaneous solubilization & refolding	SV 40 T antigen peptide tyrosine hydroxylase	Adsorption (IEX or HIC) in 50 mM Tris-HCl, pH 8.0	Q Sepharose Fast Flow	
	Human interleukin-2		Phenyl Sepharose High Performance	
	Retroviral v-myb oncoprotein			14

* HR 10/30 columns have been replaced by Tricorn high-performance 10/300 GL columns, see ordering information

Ordering information

Prepacked columns	Quantity	Code no.
Superdex Peptide 10/300 GL*	1 × 24 ml	17-5176-01
Superose 12 10/300 GL*	1 × 24 ml	17-5173-01
Superdex 75 10/300 GL*	1 × 24 ml	17-5174-01
Superdex 200 10/300 GL*	1 × 24 ml	17-5175-01
HiPrep™ 16/60 Sephacryl S-100 HR	1 × 120 ml	17-1165-01
HiPrep 26/60 Sephacryl S-100 HR	1 × 320 ml	17-1194-01
HiPrep 16/60 Sephacryl S-200 HR	1 × 120 ml	17-1166-01
HiPrep 26/60 Sephacryl S-200 HR	1 × 320 ml	17-1195-01
HiPrep 16/60 Sephacryl S-300 HR	1 × 120 ml	17-1167-01
HiPrep 26/60 Sephacryl S-300 HR	1 × 320 ml	17-1196-01
HiPrep 16/10 Q FF	1 × 20 ml	17-5190-01
HiPrep 16/10 SP FF	1 × 20 ml	17-5192-01
HiLoad™ 16/10 Phenyl Sepharose HP	1 × 20 ml	17-1085-01
HiLoad 26/10 Phenyl Sepharose HP	1 × 53 ml	17-1086-01

Prepacked columns	Quantity	Code no.
HiLoad 16/60 Superdex 30 pg	1 × 120 ml	17-1139-01
HiLoad 26/60 Superdex 30 pg	1 × 320 ml	17-1140-01
HiLoad 16/60 Superdex 75 pg	1 × 120 ml	17-1068-01
HiLoad 26/60 Superdex 75 pg	1 × 320 ml	17-1070-01
HiLoad 16/60 Superdex 200 pg	1 × 120 ml	17-1069-01
HiLoad 26/60 Superdex 200 pg	1 × 320 ml	17-1071-01

Media

Sephadex G-100	100 g	17-0060-01
Sepharose CL-6B	1 l	17-0160-01
Sephacryl S-100 HR	750 ml	17-0612-01
Sephacryl S-200 HR	750 ml	17-0584-01
Sephacryl S-300 HR	750 ml	17-0599-01
Sephacryl S-400 HR	750 ml	17-0609-01
Sephacryl S-500 HR	750 ml	17-0613-01
Phenyl Sepharose High Performance	75 ml	17-1082-01
Q Sepharose Fast Flow	300 ml	17-0510-01
SP Sepharose Fast Flow	300 ml	17-0729-01

* Tricorn columns have Valco fittings for ÄKTAdesign™ systems and are supplied with M6 connectors for FPLC System

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GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5, D-79111 Freiburg, Germany

GE Healthcare UK Limited
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327,
Piscataway, NJ 08855-1327, USA

GE Healthcare Bio-Sciences KK
Sanken Bldg. 3-25-1, Hyakunincho Shinjuku-ku
Tokyo 169-0073, Japan

Asia Pacific Tel: +85 65 62751830 Fax: +85 65 62751829 • Australasia Tel: +61 2 8820 8299 Fax: +61 2 8820 8200 • Austria Tel: 01 /57606 1613 Fax: 01 /57606 1614 • Belgium Tel: 0800 73 890 Fax: 02 416 8206 • Canada Tel: 1 800 463 5800 Fax: 1 800 567 1008 • Central, East, & South East Europe Tel: +43 1 972 720 Fax: +43 1 972 722 750 • Denmark Tel: +45 70 25 24 50 Fax: +45 45 16 2424 • Eire Tel: 1 800 709992 Fax +44 1494 542010 • Finland & Baltics Tel: +358 9 512 3940 Fax: +358 9 512 39439 • France Tel: 01 69 35 67 00 Fax: 01 69 41 98 77 • Germany Tel: 0800 9080 711 Fax: 0800 9080 712 • Greater China Tel: +852 2100 6300 Fax: +852 2100 6338 • Italy Tel: 02 26001 320 Fax: 02 26001 399 • Japan Tel: 81 3 5331 9336 Fax: 81 3 5331 9370 • Korea Tel: 82 2 6201 3700 Fax: 82 2 6201 3803 • Latin America Tel: +55 11 3933 7300 Fax: +55 11 3933 7304 • Middle East & Africa Tel: +30 210 96 00 687 Fax: +30 210 96 00 693 • Netherlands Tel: 0800-82 82 82 1 Fax: 0800-82 82 82 4 • Norway Tel: +47 815 65 777 Fax: +47 815 65 666 • Portugal Tel: 21 417 7035 Fax: 21 417 3184 • Russia & other C.I.S. & N.I.S Tel: +7 495 956 5177 Fax: +7 495 956 5176 • Spain Tel: 902 11 72 65 Fax: 935 94 49 65 • Sweden Tel: 018 612 1900 Fax: 018 612 1910 • Switzerland Tel: 0848 8028 10 Fax: 0848 8028 11 • UK Tel: 0800 515 313 Fax: 0800 616 927 • USA Tel: +1 800 526 3593 Fax: +1 877 295 8102

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